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Michael E. Connors
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Brian Seed and Yi Yang	Confirmation No.	4930
Serial No.:	10/521,634	Art Unit:	1632
371(c) Date:	October 11, 2005	Examiner:	Michael C. Wilson
Customer No.:	21559		
Title:	METHODS FOR THE PRODUCTION OF CELLS AND MAMMALS WITH DESIRED GENETIC MODIFICATIONS		

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DECLARATION UNDER 37 C.F.R. § 1.131 OF DR. BRIAN SEED

1. I am a named co-inventor of claims 1-12, 18, and 19 of the above-identified application, and I am a Professor at the Harvard Medical School and the Massachusetts General Hospital. The Massachusetts General Hospital Corporation is the Assignee of the above-identified application.

2. Prior to September 15, 2001, I and the co-inventor, or individuals under my supervision carried out experiments that are described in the attached notebook pages

(Exhibit 1; all dates have been redacted from Exhibit 1). Exhibit 1 describes the experimental methods used for characterization by fluorescence in situ hybridization (FISH) of ES cells into which an artificial chromosome has been inserted according to the methods described in the present application. In particular, these experiments were carried out by inserting into a mammalian cell an artificial chromosome containing a cassette that includes first and second regions of homology having at least 90% sequence identity to first and second regions of an endogenous chromosome of the mammalian cell and a selectable marker under conditions that result in homologous recombination between the artificial chromosome and the endogenous chromosome, resulting in integration of the cassette into the endogenous chromosome of the mammalian cell. As described in the specification (for example, at page 39, line 25, to page 40, line 29), and as illustrated in Figure 12 (copy enclosed as Exhibit 2) of the specification as filed, FISH analysis was used to confirm the proper integration of the cassette in ES cells. Exhibit 1 describes the preparation of ES cells for FISH analysis, including hybridization with a probe specific for the inserted cassette, thereby confirming that a genetically modified mammalian cell had been produced in accordance with the presently claimed methods.

3. The above experiments were carried out in the United States and completed before September 15, 2001.

4. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: August 10, 2008

Dr. Brian Seed

FISH

1, Fix cells.

[illegible] PBS. CSK. CSK+0.5% Triton — 4°C
4% paraformaldehyde (pH 7.4) — R/T
autoclave Tweezer. → check [illegible].

- slides in PBS 5min. 4°C
- CSK buffer (cold) 1 min.
- ice-cold CSK+0.5% Triton. 1 min.
- ice-cold CSK 1 min.
- 4% paraformaldehyde 10min. R/T.
- 70% EtOH 5min.
- 70% EtOH O/N. 4°C.

[illegible]: [illegible]

2, Preparation of Probe.
- denature at 75°C 10 min.
- preanneal at 42°C 20min ~ [illegible]

3, Preparation of Slides
[illegible]: 70% formamide. 2X SSC

- dehydrate slide
  - 80% EtOH 5min.
  - 95% EtOH 5min.
  - 100% EtOH 5min.
- Denature DNA (slides) in 70% formamide, 2×SSC at 75~80°C 10 min.

[illegible]. 1, [illegible] 75°C [illegible].
2, Before put slide in. Warm up slide at 50°C heat block for 2min.

- in ice-cold EtOH:
  - 70% EtOH 2 min.
  - 80% EtOH 2 min.
  - 100% EtOH 2 min.
  - 100% EtOH 2 min.
- air dry slide at R/T.

4. Hybridization.
   - warm slide at 40°C Heat Block.
   - pipette 4 μl probe DNA (2 ng/λ) on to each well.
     注意：1, 不要让它干在well上.
     2, slide一直放在40°C Heat block.
   - Cover w/ cover slip.
   - put in tissue culture incubate 37°C. O/N.

5. Wash.

   注意：1小时前 pre warm Washer Bath. [illegible]
   - ~~50% formamide 2XSSC 3X 10min 45°C~~
   - ~~2X SSC 3X 10min 45°C~~

- 3X 50% formamide 2XSSC 45°C shack at water bath 10min.
- 3X 2XSSC 45°C 10 min.
- Block at 4X SSC 0.1% Tween20 R/T 10 min.
- 150 μl of 1% BSA (use 100X BSA) 4X SSC 0.1% Tween20 with 1:50 (Fab fragments) 30 min. 37°C
- wash 3X 10 min. 45°C w/ 4XSSC 0.1% Tween20.
- add 2 μl DAPI into 50 ml 4XSSC. slide in for 2 min
- wash 1X 4XSSC R/T 2 min.

EXHIBIT 1

FISH

1. Fix cells.
   - add 5ml of trypsined ES cells on each wells of slide.
   - Air dry (≥ 20 min.)
   - slide in PBS R/T 5 min
   - 4% paraformaldehyde 10 min. R/T
   - 70% EtoH 5 min. R/T
   - 70% EtoH O/N 4°C

2. Hybridization.
   - probe
     - denecture probe at 75°C 10 min
     - preamed at 42°C (random Labeling) > 20 min.
       or at 37°C (nick translation labeling) > 20 min
   - Slides.
     - dehydrate slide.
       80% EtoH → 95% EtoH → 100% EtoH 5 min.
       5 min each at R/T
     - denature DNA slide.
       in 70% formamide 2XSSC.
       75~80°C 10 min.
     - dehydrate again. in ICE COLD EtOH.
       ice cold 70% EtoH → 80% EtoH - 95% EtoH → 100% EtoH → 100% (2X)
       2 min. each at R/T
     - Air dry slide at R/T
     - warm dry slide on 40°C heat Blk.
     - pipette 3~4ml of probe to each well.
     - cover w/ cover slip.
     - leave at tissue culture incubate 37°C O/N.

3. Wash.
   3X 2X SSC 72°C 5~7 min.
   1X 0.2X SSC 72°C 5~7 min.

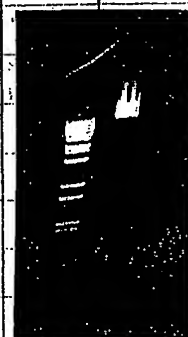
EXHIBIT 1

- Digest pNL5 (mFTIV 5' BglII-tag (≈10kb)) with BglII

100 ml RxN.

10 ml 10X High Buffer
20 ml pNL5 (1 ug/λ)
10 ml BglII (400 unit/λ)
60 ml H2O
100 ml

37°c 2 hours



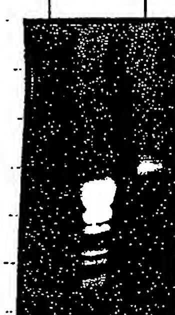
- add 100 ml phenol: mix
- spin down
- add EtOH/NaOAc
- R/S in 40 ml TE → 300 ng/λ

- Digest Sam68 - SalI religat plasmid with SalI

100 ml RxN.

10 ml 10X High Buffer
3.5 ml Sam68 - Sal religat DNA (5.9 ug/λ)
10 ml SalI (10 unit/ml)
77 ml H2O
100 ml

37°c O/N.



- add 100 ml phenol
- spin down
- 1:1 EtOH/NaOAc
- R/S in 40 ml TE → 150 ng/λ

EXHIBIT 11

FISH probe labeling (Jeannie's protocol)

- 5X Reaction Buffer
  - 92 ml 5X nucleotide buffer
  - 8 ml dig-11-dUTP

- Rxn mix

1, Jeanie's probe (15Kb)
- 10 ul primer
- 300ng 15 ml DNA (20 ng/λ)
- 14 ml H_2O
- 39 λ

2, Sam 68 pac
- 10 ul primer
- 200ng 1 ml Sam 68 pac (0.4 ng/λ)
- 28 ml H_2O
- 39 λ

3, FTH pac
- 10 ul primer
- 0.5 ml FTH pac (0.6 ng/λ)
- 300ng 28.5 ml H_2O
- 39 λ

4, FTH (Bglii-Eag 5'), ~10Kb
- 10 ul primer
- 300ng 1 ml FTH (0.3 ng/λ)
- 28 ml H_2O
- 39 λ

- heat at 100°C 5 min.
- Spin down on ice

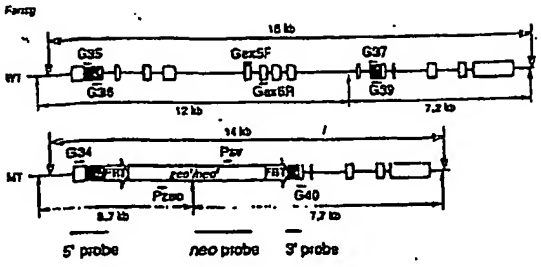
- add 10 ml 5X Reaction Buffer
  - 2 ml Klenow
  - 37°C 30 min.

- add 2 ml stop mix
- add 1:50 (probe : Cot1 DNA) Cot1 DNA. (20 ul → 15 ml)
- add 2 vol. of EtOH/NaOAc (1400 ml of EtOH/NaOAc)
  - -20°C o/n

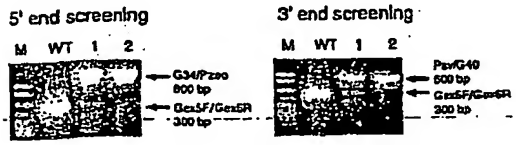
- Spin down. Air dry.

- R/S in 150 ~ 200 ul FISH Hybridization Buffer (2 ng/λ Final Conc

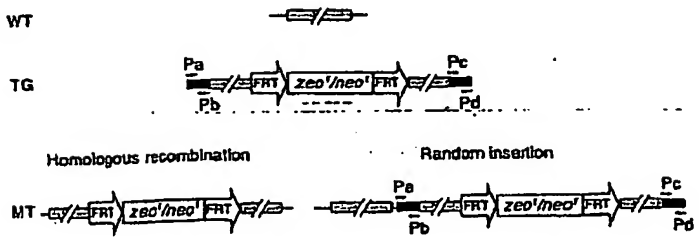
11A



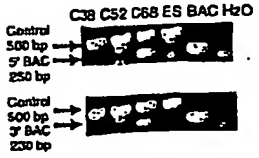
11B



12A



12B



12C